Journal of Surgical Oncology 1997;65:188–193

Tumor Invasion and Angiogenesis in Early Esophageal Squamous Cell Carcinoma

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Background and Objectives: Although the relationship between angiogenesis and tumor proliferation or malignant potential has been previously demonstrated in several studies, early stage of cancer invasion and angiogenesis has seldom been investigated.
Methods: From the esophageal specimens of eight recently resected cases with esophageal squamous cell carcinoma, 25 areas of carcinoma-in-situ or microinvasive carcinoma were selected, and then a serial histologic investigation and immunohistochemical staining for the detection of Factor VIII-related antigen to investigate microvessels in the lamina propria mucosae beneath the lesions as a measure of angiogeneses and staining for laminin to visualize basement membrane was performed. Lymphocyte infiltration below the lesions were also estimated. In view of early cancerous invasion, histologic patterns of the growth of the lesions were divided into ''flat,'' ''expansive,'' and ''downgrowth'' patterns.
Results: Although downgrowth patterns are thought to be more invasive, relationships between the histologic patterns, and basement membrane staining patterns, and lymphocyte infiltration patterns were not demonstrated. However, the angiogenetic ratio (the number of vessels/cm under the lesions divided by that under normal epithelium) was observed to be significantly and closely related to tumor invasion patterns ($P < 0.01$), although it was not related to the destruction of the basement membrane or lymphocyte infiltration below the lesions.
Conclusions: The angiogenesis of esophageal squamous cell carcinoma is closely correlated to the tumor invasion patterns in early esophageal cancerous lesions. *J. Surg. Oncol. 1997;65:188–193.*

KEY WORDS: angiogenesis; tumor invasion; Factor VIII-related antigen; esophageal cancer

INTRODUCTION

Esophageal squamous cell carcinoma has a unique carcinogenesis [1,2] and growth pattern [3,4]. The existence of intraepithelial carcinoma contiguous to the main lesions is not a rare event [5], and such preinvasive lesions often present multicentrically [6]. Nagamatsu et al. [7] reported that lymphocytic infiltration was found to be located farther beneath the intraepithelial carcinoma than below the normal mucosa of the esophagus. Baba et al. [8] reported that superficial esophageal cancer might progress while destroying the basement membrane. Thereafter, the lymphocytes might infiltrate the lesions where the basement membranes have been destroyed, and the cancer cells would thus be exposed to the underlying layer. More recently, Sadanaga et al. [9] demonstrated the results of an immunohistochemical study of human leukocyte antigen HLA-DR and lymphocyte infiltration and suggested that the local immune response

Contract grant sponsor: Japanese Ministry of Education; Contract grant number: 4091; Contract grant sponsor: Fukuoka Cancer Society.
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Accepted 30 March 1997

to the HLA-DR may thus prevent tumor invasion, whereas the negative expression of HLA-DR antigen is a significant factor facilitating tumor invasion in esophageal cancer.

There have been reports concerning the tumor invasion of esophageal cancer. Shima et al. [10] reported that matrix metalloproteinases may play an important role in tumor invasion. In addition, Doki et al. [11] reported that the reduction of E-cadherin adhesion molecule expression may affect the mode of invasion. These biological characteristics of cancer cells are thus considered to be significant indicators of tumor invasion.

However, it is now well established that invasive tumors depend on neovascularization for their continued growth, expansion, and possibly metastasis [12]. Several angiogenetic molecules have been identified and shown to be produced by tumors [12,13]. The correlation between angiogenesis and tumor proliferation [14] or malignant potential [15–17] also has been well demonstrated in the literature. However, to our knowledge, there have been few investigations of angiogenesis in esophageal cancer [14,18], and no demonstration of the relationship between angiogenesis and tumor invasion has been reported. We, therefore, analyzed the vascular distribution of human esophageal cancer and its relationship, especially in regard to tumor invasion.

MATERIALS AND METHODS

Patients and Tumor Specimens

Sixty-seven patients with primary esophageal carcinoma underwent esophagectomy without preoperative treatment in the Department of Surgery II, Kyushu University, between 1991 and 1993. Microscopic sections of the whole resected esophagus were made from step-sectioned blocks measuring 0.5 cm in width and stained with hematoxylin and eosin (H&E).

In order to study the angiogenesis around the tumor among these 67 cases, eight cases of esophageal carcinoma containing the areas of either intraepithelial carcinoma or microinvasive carcinomas measuring >1.0 cm in length were selected for these studies. As a result, 25 different lesions of intraepithelial carcinoma or microinvasive carcinoma were finally selected for this study.

Immunohistochemical Procedure

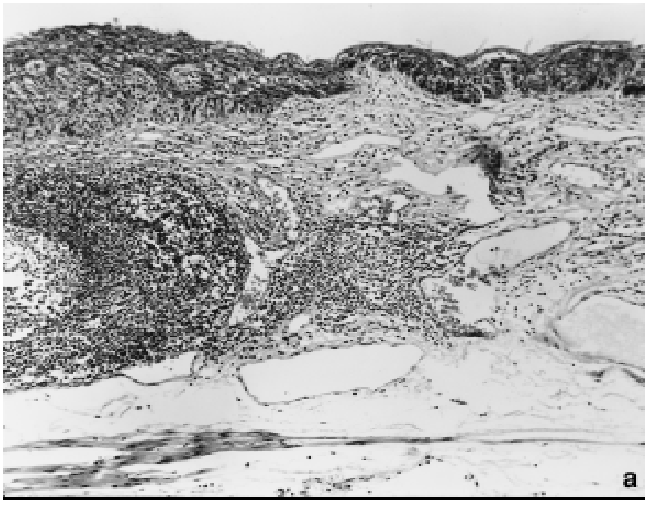
The streptavidin biotin method was applied. In brief, after the sections were dewaxed, the endogenous peroxidase activity was inactivated in 100% methanol with 0.3% hydrogen peroxide. The sections intended for the study of von Willebrand factor (Factor VIII-related antigen) were prepared with trypsin, whereas those for laminin were prepared with pepsin. The sections were incubated for 30 minutes with normal nonimmune serum to block the cross-reactivity. Any excess normal serum was removed and replaced by the primary antibodies: rabbit antihuman von Willebrand factor (dilution 1:500; A082, Dacopatts, Glostrup, Denmark) and mouse antihuman laminin (dilution 1:100; F-54, Fuji Chemical Industries, Toyama, Japan). The sections were incubated overnight at 4°C with these primary antibodies in a humid chamber. After the sections were washed, they were incubated with a biotin-labeled secondary antibody for 30 minutes and peroxidase-labeled streptavidin for 20 minutes, followed by the staining with 0.03% 3′3-diaminobenzidinetetrahydrochloride (DAB) prepared in 0.01 M phosphate-buffered saline (PBS) containing 0.01% hydrogen peroxide, and counterstained with methyl green. Then they were dehydrated and mounted for a microscopic examination.

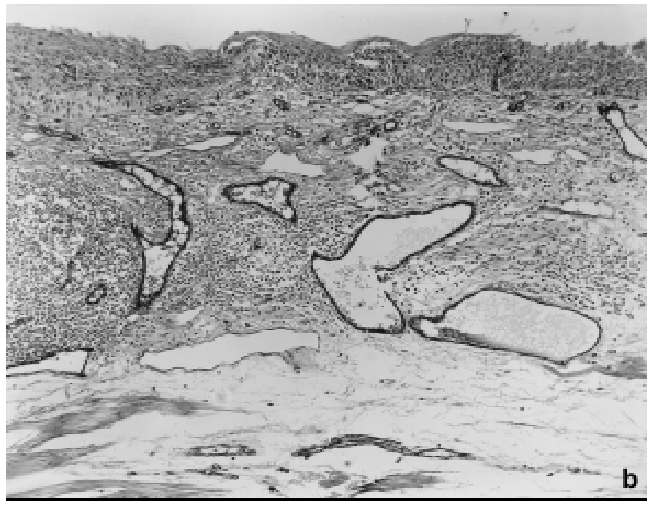
The number of factor VIII-staining structures within the lamina propria mucosae underlying either the carcinoma-in-situ or microinvasive carcinoma and normal esophageal squamous epithelium adjacent to the lesions was counted and the length of the basement membranes were measured. The values were expressed as vessel/cm of normal and carcinoma-in-situ or microinvasive cancerous epithelium. The angiogenetic ratio for each lesion is the number of vessels/cm under the lesion divided by the number of vessels/cm under the normal epithelium [19].

These patterns were also analyzed for any correlation with the degree of lymphocyte infiltration either below or near the basement membrane. The infiltrative tendency of the lymphocytes was also divided into three categories as follows; (±): lymphocyte infiltration, which is no denser than in the mucosa with the background normal epithelium, (+): lymphocyte infiltration denser than the background but lacking follicle formation, and (++): dense lymphocyte infiltration with follicle formation [7]. The staining patterns of the basement membrane were also classified into continuous and destructive patterns [8].

The growth patterns of carcinoma-in-situ or microinvasive carcinoma were also classified into three patterns; flat type (Fig. 1), expansive type (Fig. 2), and downgrowth patterns (Fig. 3). The histologic identification of either noninvasive or invasive lesions of the early esophageal lesions (carcinoma-in-situ or microinvasive cancer) is quite difficult even using immunohistochemical staining of the basement membrane. Therefore, we originally used the growth pattern of early esophageal lesions: the flat type was defined as a lesion that was basically similar to adjacent noncancerous epithelium; the expansive type was characterized by bulky continuous growth of the lesions, and the downgrowth type was shown as a discontinuous downgrowth pattern in the slides. Nevertheless, the lesions with a downgrowth pattern were thought to be more invasive in nature than the others.

The extent of the 25 areas was essentially judged based on the growth pattern of these lesions.





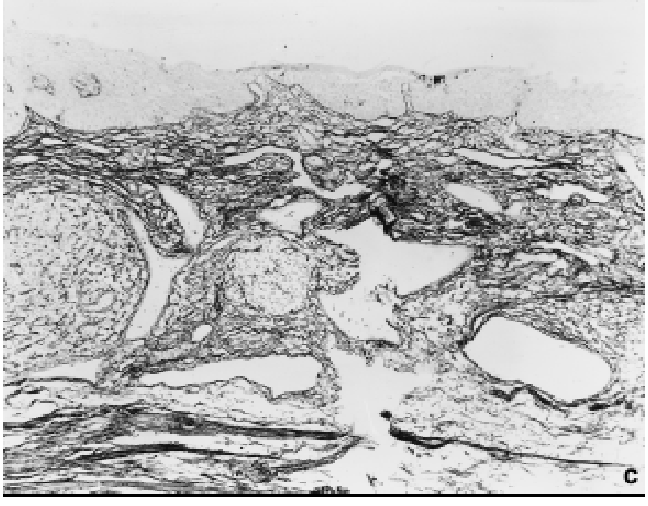


Fig. 1. **(a)** This histology of flat-type esophageal carcinoma (H&E, ×80). **(b)** Factor VIII-related antigen staining. Little vasculature was observed within the epithelial lesions in spite of an abundant number of dilated subepithelial vessels (ABC method, ×80). **(c)** Laminin staining. The basement membrane of the epithelium was continuously preserved (ABC method, ×80).

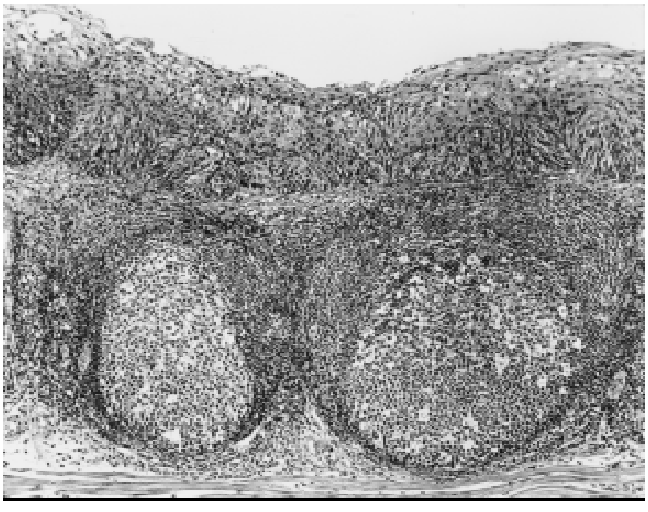


Fig. 2. The histology of expansive-type growth. There were lymphoid follicles beneath the lesions (H&E, ×80).

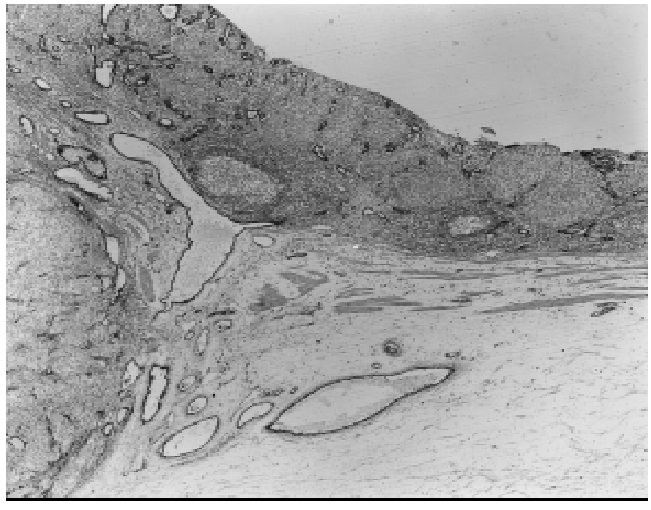


Fig. 3. Factor VIII-related antigen staining of downgrowth type of carcinoma. A large number of vessels was observed beneath the early cancerous lesion, as well as at the more invasive site (left side). Blood vessel counting was done only beneath the microinvasive lesion restricted within the mucosal layer (right side) (ABC method, ×27).

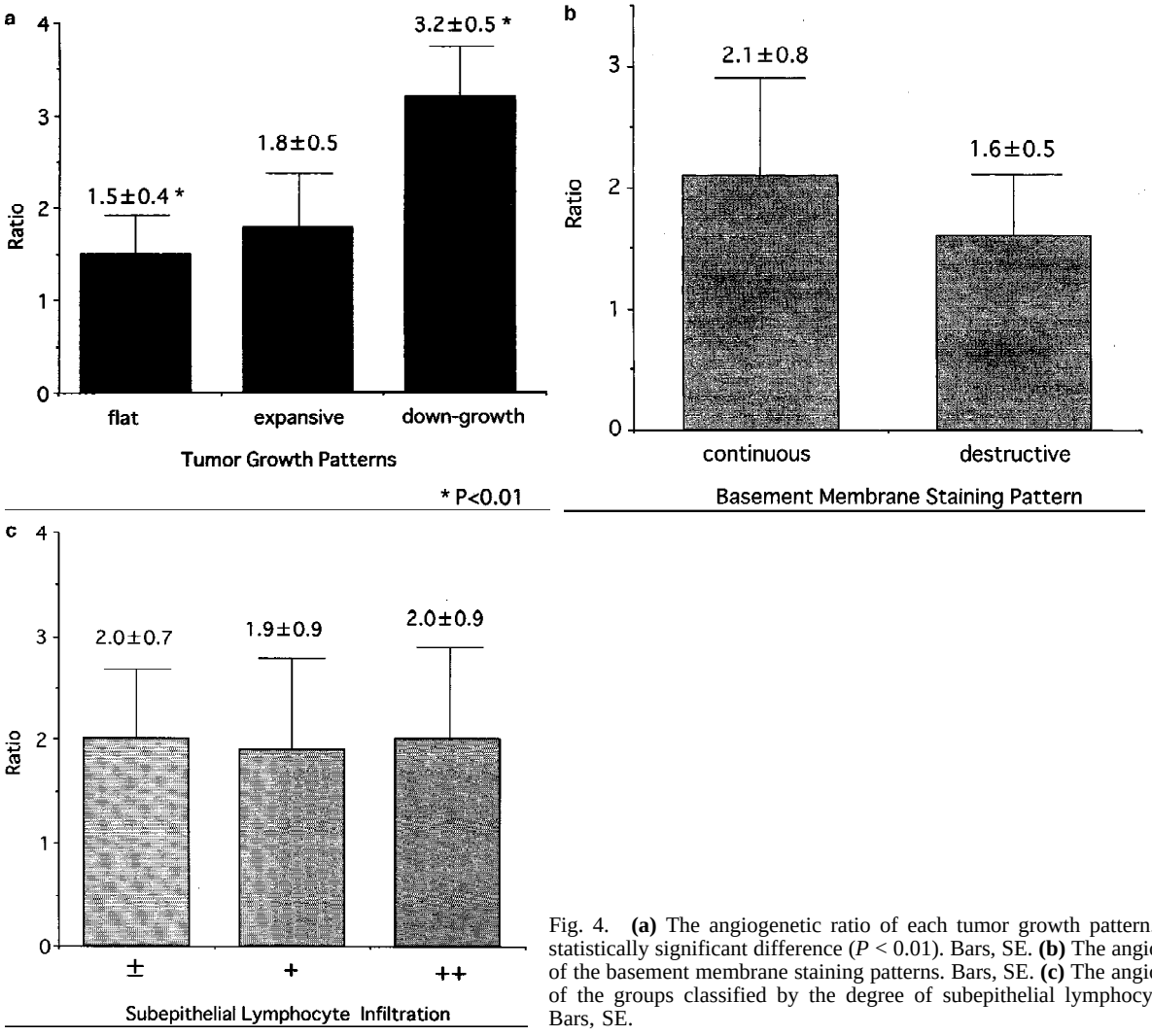


Fig. 4. **(a)** The angiogenetic ratio of each tumor growth pattern. There was a statistically significant difference ($P < 0.01$). Bars, SE. **(b)** The angiogenetic ratios of the basement membrane staining patterns. Bars, SE. **(c)** The angiogenetic ratios of the groups classified by the degree of subepithelial lymphocyte infiltration. Bars, SE.

Statistical Analysis

The results of the immunohistochemical study were analyzed using the Chi-square test. The difference was considered significant when the *P*-value was <0.05.

RESULTS

The histopathology of flat, expansive, and downgrowth patterns in H&E, Factor VIII, and laminin staining are shown in Figures 1, 2, and 3, respectively. In the flat type, small neovascularized vessels were scanty, although several large blood vessels existed (Fig. 1b). In the expansive type, small blood vessels existed both within and without lymphoid follicles in the lamina propria mucosae. In the downgrowth type, neovascularization was most prominent beneath the lesions as well as at more invasive sites in the submucosal layer (Fig. 3) and the blood vessel count was done only beneath the areas of the microinvasive sites.

A comparison of the vessel counts of carcinoma-in-situ or microinvasive cancerous lesions with the counts of adjacent normal tissue showed a statistical difference ($P < 0.0001$). Since there was a wide range in the values for both the normal tissue and the lesions, the data were also expressed as a ratio of the vessel count of the lesion divided by the normal epithelium from the same specimen (angiogenetic ratio). The difference in the angiogenetic ratios of the three types of histologic tumor growth patterns was statistically significant ($P < 0.01$) (Fig. 4), whereas the difference in the angiogenetic ratios between the groups classified according to the basement membrane staining patterns, or between the three groups of lymphocyte infiltration showed no statistically significant difference (Fig. 4b,c).

In contrast, there were no close relationships between the tumor invasion patterns and basement membrane staining pattern or subepithelial lymphocyte infiltration (Table I). Thus in this study, the relationship only between the tumor growth patterns of the carcinoma-in-situ or microinvasive carcinoma and angiogenetic ratio was demonstrated.

TABLE I. Tumor Growth Pattern, Basement Membrane Staining Pattern, and Degree of Subepithelial Lymphocyte Infiltration

Tumor invasion patterns	Basement membrane staining pattern		Subepithelial lymphocyte infiltration		
	Continuous	Destructive	±	+	++
Flat (n = 10)	6	4	5	4	2
Expansive (n = 10)	8	2	5	3	2
Downgrowth (n = 5)	4	1	2	2	1

DISCUSSION

Concerning the mechanism of the early invasion of squamous cell carcinoma, it is still questionable as to whether or not the staining of the basement membrane is useful. One of the important factors contributing to the degeneration of the basement membrane are inflammatory cells such as macrophages and polymorphonuclear leukocytes, which secrete proteolytic enzymes. The infiltration of these cells has been described in relation to the defects of the basement membrane in intraepithelial neoplasia of the larynx and uterine cervix [20–22]. We recently performed immunohistochemical staining on the basement membrane in early esophageal carcinoma, and the results demonstrated that neither any macrophages nor polymorphonuclear leukocytes were observed to a substantial degree, and thus the lymphocytes tended to infiltrate regions in which the basement membrane had been destroyed. These findings may thus provide further evidence in support of the hypothesis that lymphocyte infiltration might be one of the immune defense mechanisms of the host against carcinoma [8]. However, it is still possible that these infiltrating lymphocytes might also accelerate the destruction of the basement membrane, because lymphocytes have the potential to produce stimulating factors that enhance the collagenolytic activity of normal fibroblast populations [23]. It is a fact that a close relationship exists between the destruction of the basement membrane and lymphocyte infiltration below early esophageal carcinomatous lesions. Based on these findings, we first investigated the relationship between tumor angiogenesis and either the destruction of the basement membrane or lymphocyte infiltration. However, no relationship between them was demonstrated in this study.

We thus paid careful attention to the growth pattern of early esophageal cancer and its relation to angiogenesis. Based on the results of ordinary hematoxylin & eosin staining, it is very difficult to evaluate the degree of destruction of the basement membrane, since the basement membrane sometimes exists even in deeply invasive cancer tissue. Thus the distinction between carcinoma-in-situ (intraepithelial carcinoma) and microinvasive carcinoma (mucosal carcinoma) is quite difficult even when basement membrane staining is done. The growth pattern of early esophageal cancerous lesions is considered to be one of the most reliable features for evaluating the early invasive nature of carcinoma. In this study, no relationship was found between the angiogenetic ratio or tumor growth pattern and basement membrane staining or lymphocyte infiltration. The destruction of laminin is thought to be more prominent in the lesions located at more invasive sites, such as the proper muscular layer or the adventitia, and the lesions within the lamina propria mucosae investigated in this study were in too early a stage to demonstrate any difference in the laminin staining patterns. However, subepithelial lymphocyte infiltration existed even in the dysplastic epithelium [7], the difference in the lymphocyte infiltration pattern in each lesion of either carcinoma-in-situ or microinvasive carcinoma thus could not be demonstrated. However, the tumor growth pattern and the angiogenetic ratio was shown to be closely related, and based on our findings, angiogenesis within carcinoma-in-situ or microinvasive cancerous lesions was thus found to be prominent in the areas of more invasive features. This finding also is considered to be helpful in the investigation of tumor angiogenesis.

For further study of tumor angiogenesis, an investigation of the expression of angiogenic growth factor, such as vascular endothelial growth factor (VEGF) [24], in the tumor cells would thus be helpful to clarify the relationship between tumor growth and angiogenesis.

ACKNOWLEDGMENTS

The authors thank Mr. Brian T. Quinn for critical comments.

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